Beautiful modifications made simple.

MetaMorph Mutagenesis Kit
Cat. #MC200 Series

User Manual

Store competent cells at -80°C
Store enzymes at -20°C

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ver. 1-110110

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I. Introduction

The MetaMorph Mutagenesis Kit is a simple, rapid and highly efficient PCR-based mutagenesis kit. It allows you to create single and multiple point mutations, deletions or insertions using an easy one-day protocol. Mutagenic PCR fragments are generated with a high fidelity DNA polymerase, mutagenic primers, and vector primers. The mutagenic primers are designed to incorporate the mutation, insertion or deletion. Vector primers are designed to have 20bp homology to the linearized vector ends. The mutagenic PCR fragments fuse to create the final mutated gene-of-interest. The kit is so robust that multiple mutagenic DNA fragments can be assembled simultaneously and cloned into one construct in a single step. The system is highly efficient, with more than 90% efficiency for single mutations, without unwanted mutations being introduced.

A. Key Features

- Effective for both single and multiple mutation sites
- Introduce point mutations, deletions, or insertions
- Complete mutagenesis within one day
- Only one transformation step required
- May be used with any vector
- Methylation-independent
- Compatible with all strains of competent *E. coli* cells
- Unwanted additional mutations are minimized

B. List of Components

Kit Component	10 Reactions
High Fidelity DNA Polymerase	5 µl
5x PCR Buffer	100 µl
Control primer 1 (10 µM)	25 µl
Control Primer 2 (10 µM)	25 µl
Control Primer 3 (10 µM)	25 µl
Control Primer 4 (10 µM)	25 µl
Control Primer 5 (10 µM)	25 µl
Control Primer 6 (10 µM)	25 µl
Control Plasmid (0.5 mg/ml)	10 µl
dNTP mix (10mM)	10 µl
5x MetaMorph Solution	20 µl
Competent Cells	10 vials

C. Storage

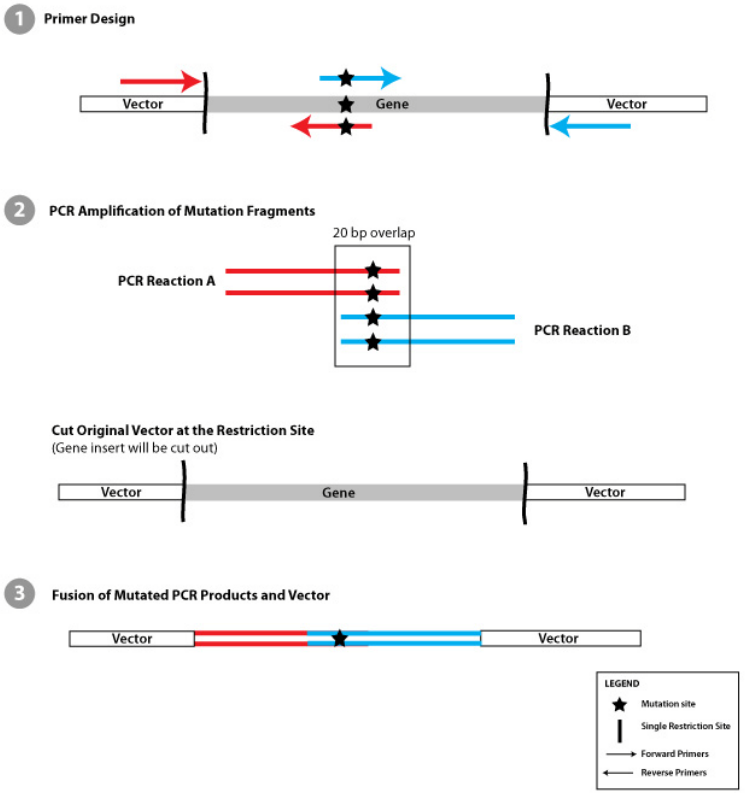
-80°C for competent cells

-20°C for the enzyme

D. Other Needed Reagents

- Gene-specific primers
- DMSO
- QIAquick PCR Purification Kit (Cat # 28106, Qiagen)
- QIAquick Gel Extraction kit (Cat # 28704, Qiagen)
- SOC or LB Broth for transformation of bacteria
- LB + Antibiotic plates (depending upon your specific plasmid)

E. Overview of Protocol



F. One-day Protocol

9:00 am – 1:00 pm	PCR Amplification of mutations and restriction digest of vector
1:00 – 2:00 pm	Run gel, gel purify PCR products and linearized vector
2:00 – 2:30 pm	Reaction Incubation
2:30 – 4:00 pm	Transformation

II. Protocols

A. Primer Design

1. Generating Single Point Mutations or Insertion Mutations

Forward and Reverse Vector Primer Design

The forward vector primer (in red) should be generated to the positive strand of DNA, and should contain ~20 bases corresponding to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.

The reverse vector primer (in blue) should be generated to the negative strand of DNA, and should also contain ~20 bases complimentary to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.

Mutagenic Primers

The reverse mutagenic primer (in red) should be generated to the negative strand of DNA, and should contain 10 bases 5' of the mutation, the mutagenic bases, and then 20 bases 3' of the mutation site.

The forward mutagenic primer (in blue) should be generated to the positive strand of DNA, and should contain 10 bases 5' of the mutation, the mutagenic or inserted bases, and then 20 bases 3' of the mutation site.

The forward and reverse mutagenic primers, therefore have a region of ~20 bases that is entirely complimentary and that contains the mutagenic bases.

Melting Temperature of Primers for Point Mutations and Insertion Mutations

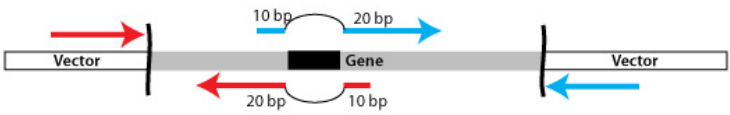
The T_m for primers for point mutations and insertion mutations should be calculated using only the 20 bases that are 3' of the mutagenic site. The mutagenic base(s) and the 10 bp that are 5' of the mutation site should not be included in the calculation for T_m.

2. Generating Deletion Mutations

Forward and Reverse Vector Primer Design

The forward vector primer (in red) should be generated to the positive strand of DNA, and should contain ~20 bases corresponding to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.

The reverse vector primer (in blue) should be generated to the negative strand of DNA, and should also contain ~20 bases complimentary to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.



Mutagenic Primers

The reverse mutagenic primer (in red) should be generated to the negative strand of DNA, and should contain 10 bases 5' of the mutation, (remove the bases you wish to delete), and then 20 bases 3' of the mutation site.

The forward mutagenic primer (in blue) should be generated to the positive strand of DNA, and should contain 10 bases 5' of the mutation, (remove the bases you wish to delete), and then 20 bases 3' of the mutation site.

The forward and reverse mutagenic primers, therefore have a region of ~20 bases that is entirely complimentary and do not contain the bases intended for deletion.

Melting Temperature for Deletion Mutation Primers

The T_m of the primers for deletion mutations should be calculated using the 20 base pairs of homology in the 3' region. The 10 base pairs that are homologous to the 5' region will not affect the T_m.

3. Generating Multiple Mutations

Forward and Reverse Vector Primer Design

The forward vector primer (in red) should be generated to the positive strand of DNA, and should contain ~20 bases corresponding to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.

The reverse vector primer (in green) should be generated to the negative strand of DNA, and should also contain ~20 bases complimentary to the vector sequence leading up to the restriction site where the gene-of-interest has been inserted.

Mutagenic Primers

The reverse mutagenic primer #1 (in red) should be generated to the negative strand of DNA, and should contain 10 bases 5' of the mutation, the mutation (base substitution or deletion), and then 20 bases 3' of the mutation site.

The forward mutagenic primer #1 (in blue) should be generated to the positive strand of DNA, and should contain 10 bases 5' of the mutation, the mutation (base substitution or deletion), and then 20 bases 3' of the mutation site.

The reverse mutagenic primer #2 (in blue) should be generated to the negative strand of DNA, and should contain 10 bases 5' of the second mutation, the mutation (base substitution or deletion), and then 20 bases 3' of the second mutation.

The forward mutagenic primer #2 (in green) should be generated to the positive strand of DNA, and should contain 10 bases 5' of the second mutation, the mutation (base substitution or deletion), and then 20 bases 3' of the second mutation.

The sets forward and reverse mutagenic primers, therefore have a region of ~20 bases that is entirely complimentary and contain the desired, respective mutations.

B. PCR Reactions and Vector Linearization

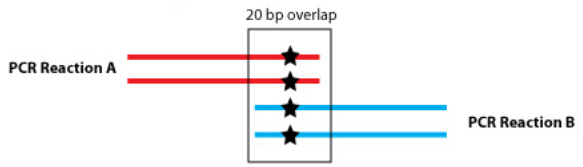
1. Single Point, Insertion, and Deletion Mutations

In a single mutation reaction, 2 PCR reactions must be set up. You can set them up and run them simultaneously.

PCR Reaction A
This reaction should contain the forward vector primer, and the reverse mutagenic primer, in addition to the template DNA and other required reagents for the PCR reaction.

PCR Reaction B
This reaction should contain the forward mutagenic primer and the reverse vector primer, in addition to the template DNA and other required reagents for the PCR reaction.

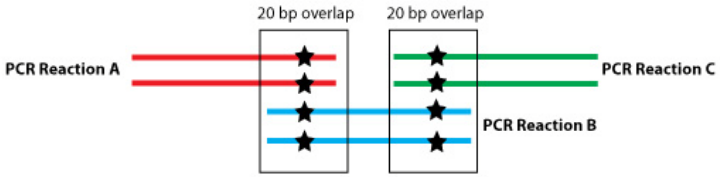
The resulting PCR reactions create 2 fragments of the mutated gene of interest. One is a 5' region of the mutated gene; the other is a 3' region of the mutated gene. The fragments have approximately 20 base pairs of overlap.



2. Multiple Mutations
In a multiple mutation reaction, the number of PCR reactions equals one more than the number of mutations (R= n+1). For example, if you are designing a construct with 2 mutations, R=2 +1 = 3 PCR reactions must be set up. If you are designing a construct with 3 mutations, 4 PCR reactions must be set up. These can be set up and run simultaneously.

Each reaction should contain a forward primer (either the forward vector primer, or mutagenic primer), and a corresponding reverse primer (either the reverse vector or mutagenic primer).

The resulting PCR reactions create n+1 fragments of the mutated gene of interest. The most 5' reaction contains the 5' region of the mutated gene; the last reaction contains a 3' region of the mutated gene. Each fragment has approximately 20 base pairs of overlap with the neighboring fragment.



3. Other Information about PCR Conditions

- When using the MetaMorph Mutagenesis Kit for the first time, we strongly recommend that you perform the positive control reactions in parallel with your experimental samples. The PCR reactions and conditions for the positive controls can be found in Section III of this manual.

- The PCR fragments for the mutated cDNA-of-interest should be generated with the high fidelity DNA polymerase that is included in the kit. Use of a substituted DNA polymerase will decrease the efficiency of the MetaMorph Mutagenesis kit.

- Specific PCR reaction conditions should be optimized for the cDNA-of-interest.

Recommended PCR conditions for a 50 µl reaction

Component	Volume
H_2O	Add to 50 µl
5x PCR Buffer	10 µl
dNTPs (10 mM)	1 µl
Primer A (10 µM)	x µl
Primer B (10 µM)	x µl
Template DNA	x µl
DMSO	1.5 µl
High Fidelity DNA polymerase (from kit)	0.5 µl

Recommended PCR Program

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation Annealing Extension	98°C - -°C* 72°C	30s 10-30s 15-30s /kb	30
Final Extension	72°C 4°C	10 min Hold	1

*For primers with >20nt, anneal for 10-30s at 3°C plus the T_m of lower primer. For primers≤ 20nt, use an annealing temperature equal to the T_m of the lower primer.

- After completion of the PCR reaction, gel purify the appropriate band to remove any extra primers or primer dimmers that will inhibit the reaction. We recommend the QIAquick PCR Purification Kit (Cat # 28106, Qiagen).

4. Linearize the Vector

While the PCR reactions are running, the vector containing the wild-type gene-of-interest should be linearized with restriction enzyme(s) to release the gene-of-interest and should then be gel purified. We recommend using 2 µg of plasmid in a 50 µl reaction volume for the restriction digest.

C. MetaMorph Reactions

Set up the following reactions in a 1.5 ml sterile reaction tube by mixing the following reagents gently. Spin down briefly to collect the reagents at the bottom of the tube.

1. Mutagenesis Reaction

Linearized destination vector (10-100ng/μl)	1μl*
PCR insert(s) (20-200ng/μl) (for each PCR Product)	1μl*
dH_2O	_μl
5x MetaMorph Solution	2μl
total	10μl

2. Negative Control

Control vector (2ug) cut with Nde1+BamH1	1μl*
dH_2O	7μl
5x MetaMorph Solution	2μl
total	10μl

* A 2:1 or 1:1 molar ratio of insert: vector works well in the MetaMorph reaction.

For reactions with larger volumes of vector and insert (>8μl of vector + insert), double the amount of reaction buffer and enzyme, and add dH_20 for a total volume of 20μl.

When using the MetaMorph Mutagenesis Kit for the first time, we strongly recommend that you perform the positive and negative control reactions in parallel with your experimental samples. ***The specific reaction conditions are found in Section III of this manual.***

3. MetaMorph Reaction Incubation

1. 10 minutes at room temperature
2. 10 minutes one ice

D. Transformation

1. Add 50μl competent cells to the cloning mixture
2. Incubate on ice for 20 minutes
3. Heat shock at 42˚C for 50 seconds
4. Transfer on ice for 2 minutes
5. Add 250μl S.O.C medium or LB broth
6. Incubate at 37˚C for an hour
7. Take 100μl culture spread on pre-warmed (37˚C) culture plate containing the appropriate antibiotic for your plasmid.
8. Incubate the plate at 37˚C overnight.

III. Examples

A. Positive Contol for Insertions

PCR Reactions Single Mutations (insertion)

PCR Reaction A	
H_2O	32 µl
5x PCR Buffer	10 µl
10mM dNTPs	1 µl
Primer 1 (10mM)	2.5 µl
Primer 2 (10mM)	2.5 µl
Template DNA	0.2 µl
DMSO	1.5 µl
High Fidelity DNA polymerase	0.5 µl

PCR Reaction B	
H_2O	32 µl
5x PCR Buffer	10 µl
10mM dNTPs	1 µl
Primer 3 (10mM)	2.5 µl
Primer 4 (10mM)	2.5 µl
Template DNA	0.2 µl
DMSO	1.5 µl
High Fidelity DNA polymerase	0.5 µl

PCR Conditions Single Mutations (insertion)

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation Annealing Extension	98°C 55°C 72°C	30s 30s 20s	30
Final Extension	72°C 4°C	10 min Hold	1

Linearize the Control Vector

H_2O	40 µl
10x Buffer 2	5 µl
Control vector (0.5ug/ul)	4 µl
Nde 1	0.5 µl
BamH1	0.5 µl

Positive control MetaMorph reaction for single mutations

Control vector cut with Nde1+BamH1	1 µl
PCR mutation fragment A	1 µl
PCR mutation fragmentB	1 µl
dH2O	5 µl
5x MetaMorph Solution	2 µl
Total	10 µl

B. Positive Control for Multiple Mutations

PCR Reactions for Multiple Mutations

PCR Reaction A	
H_2O	32 µl
5x PCR Buffer	10 µl
10mM dNTPs	1 µl
Primer 1 (10mM)	2.5 µl
Primer 5 (10mM)	2.5 µl
Template DNA	0.2 µl
DMSO	1.5 µl
High Fidelity DNA polymerase	0.5 µl

PCR Reaction B	
H_2O	32 µl
5x PCR Buffer	10 µl
10mM dNTPs	1 µl
Primer 6 (10mM)	2.5 µl
Primer 2 (10mM)	2.5 µl
Template DNA	0.2 µl
DMSO	1.5 µl
High Fidelity DNA polymerase	0.5 µl

PCR Reaction C	
H_2O	32 µl
5x PCR Buffer	10 µl
10mM dNTPs	1 µl
Primer 3 (10mM)	2.5 µl
Primer 4 (10mM)	2.5 µl
Template DNA	0.2 µl
DMSO	1.5 µl
High Fidelity DNA polymerase	0.5 µl

PCR Conditions for Multiple Mutations

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	3 min	1
Denaturation Annealing Extension	98°C 55°C 72°C	30s 30s 20s	30
Final Extension	72°C 4°C	10 min Hold	1

Linearize the control vector

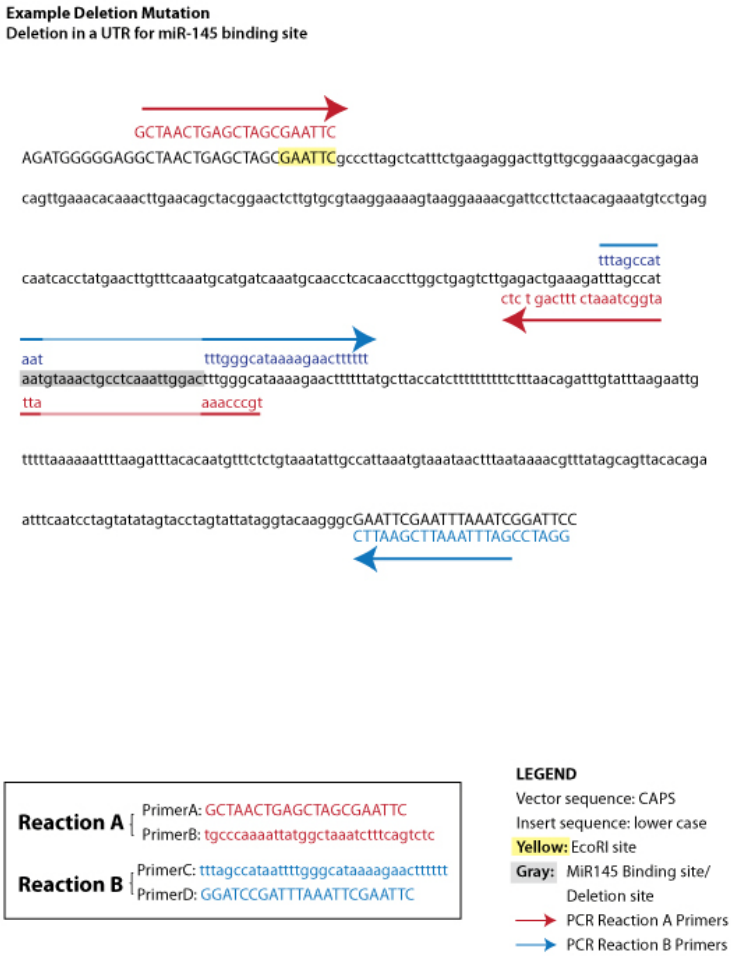
H_2O	40 µl
10x Buffer 2	5 µl
Control vector (0.5ug/ul)	4 µl
Nde 1	0.5 µl
BamH1	0.5 µl

Positive control MetaMorph reaction for multiple mutations

Control vector (2ug) cut with Nde1+BamH1	1 µl
PCR mutation fragment A	1 µl
PCR mutation fragment B	1 µl
PCR mutation fragment C	1 µl
dH2O	4 µl
5x MetaMorph Solution	2 µl
total	10 µl

C. Example Deletion Mutation

(No positive control for deletions included in the MetaMorph kit)

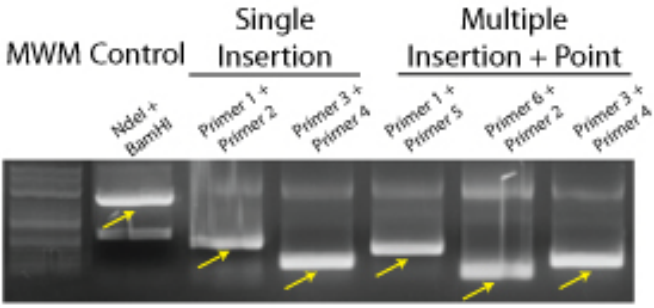


Reaction A [PrimerA: GCTAACTGAGCTAGCGAATTC
PrimerB: tgcccaaaattatggctaaatctttcagtctc

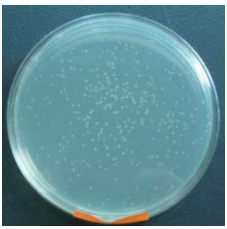
Reaction B [PrimerC: tttagccataattttgggcataaaagaacttttttt
PrimerD: GGATCCGATTTAAATTCGAATTC

LEGEND
Vector sequence: CAPS
Insert sequence: lower case
Yellow: EcoRI site
Gray: MiR145 Binding site/ Deletion site
→ PCR Reaction A Primers
→ PCR Reaction B Primers

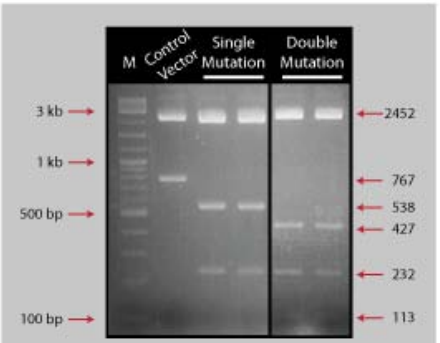
D. Sample Mutagenesis Results


MWM Control
Single Insertion
Multiple Insertion + Point
NdeI + BamHI
Primer 1 + Primer 2
Primer 3 + Primer 4
Primer 1 + Primer 5
Primer 6 + Primer 2
Primer 3 + Primer 4

The yellow arrows indicate the bands that need to be extracted from the gel. The PCR fragments are then combined with the cut vector and the 5x MetaMorph Solution to create the finished mutagenic construct. The construct is then transformed into any kind of competent *E. coli* strain.



More than 90% of the clones will contain the mutation.



Control and Mutated samples cut with NdeI and BamHI. Control vector has only 1 NdeI and 1 BamHI restriction site. The single insertion mutation adds a new NdeI site. The double mutation (insertion and point mutation) adds NdeI and BamHI sites.

IV. Troubleshooting

Problem	Probable cause	Solution
1. No or few colonies obtained from the transformation	Primer sequences are incorrect	Check primer sequences to ensure that they follow the rules of primer design as described in the manual.
	Suboptimal PCR product	Optimize your PCR amplification reactions so that you generate pure PCR products. Use a different method to purify your PCR product.
	Low DNA concentration in reaction	It is imperative to obtain as high a DNA concentration as possible in your reaction.
	Inhibitory contaminants from PCR product or linearized vector	Both the PCR products and the linearized vector should be purified.
	Transform with too much reaction mixture	Do not add more than 10μl of reaction mixture to 50μl of competent cells. Too much reaction mixture inhibits the transformation.

Problem	Probable cause	Solution
	Low quality or poor handling of competent cells	Handle competent cells gently. Do not re-freeze cells after thawing. Quality of competent cells may be tested by transforming a circular plasmid to determine cells' competency. Competent cells with a transformation efficiency of ≥ $1x10^9$ cfu/ µg are recommended.
	Wrong antibiotic or too much antibiotic in the media	Choose plates with the appropriate concentration of the right antibiotic.
2. Large numbers of colonies contain no insert	Incomplete linearization of your vector	It is critical to remove any uncut vector prior to use in the MetaMorph reaction. If necessary, re-digest your vector and gel purify.
	Contamination of cloning reaction with plasmid with the same antibiotic resistance	If you insert was amplified from a plasmid, circular DNA may have carried through purification and contaminated the cloning reaction. We recommended gel purifying your PCR product or linearizing the template DNA before performing PCR.

Problem	Probable cause	Solution
2. Large numbers of colonies contain no insert	Plates are too old or contained incorrect antibiotic	Make sure that your antibiotic plates are fresh. Check the antibiotic resistance of your fragment.
3. Clones contain incorrect insert	PCR products contain non-specifically amplified artifacts	Optimize your PCR reaction to improve the specificity. Screen more colonies for the correct clones.

V. Technical Support

For more information about SBI products and to download manuals in PDF format, please visit our web site:

http://www.systembio.com

For additional information or technical assistance, please call or email us at:

Phone: (650) 968-2200
(888) 266-5066 (Toll Free)
Fax: (650) 968-2277
E-mail:
General Information: info@systembio.com
Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

System Biosciences (SBI)
1616 North Shoreline Blvd.
Mountain View, CA 94043

VI. Licensing and Warranty

Limited Use License

Use of the MetaMorph Mutagenesis Kit (i.e., the “Product”) is subject to the following terms and conditions. If the terms and conditions are not acceptable, return all components of the Product to System Biosciences (SBI) within 7 calendar days. Purchase and use of any part of the Product constitutes acceptance of the above terms. Purchase of the product does not grant any rights or license for use other than those explicitly listed in this Licensing and Warranty Statement. Use of the Product for any use other than described expressly herein may be covered by patents or subject to rights other than those mentioned. SBI disclaims any and all responsibility for injury or damage which may be caused by the failure of the buyer or any other person to use the Product in accordance with the terms and conditions outlined herein. SBI has pending patent applications related to the Product. For information concerning licenses for commercial use, contact SBI.

Limited Warranty

SBI warrants that the Product meets the specifications described in the accompanying Product Analysis Certificate. If it is proven to the satisfaction of SBI that the Product fails to meet these specifications, SBI will replace the Product or provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to SBI within 30 days of receipt of the Product.

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